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Metzler-Zebeli, B., Magowan, E., Hollman, M., Ball, E., Molnar, A., Witter, K., ERTL, R., Hawken, R., Lawlor, P. G., O'Connell, N., Aschenbach, J., & Zebeli, Q. (2017). Differences in intestinal size, structure and function contributing to feed efficiency in broiler chickens reared at geographically distant locations. *Poultry Science*, 578-597. https://doi.org/10.3382/ps/pex332

Published in:
Poultry Science

Document Version:
Peer reviewed version

Running head: RFI-related gut function

Differences in intestinal size, structure and function contributing to feed efficiency in broiler chickens reared at geographically distant locations

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Abstract

The contribution of the intestinal tract to differences in residual feed intake (RFI) has been inconclusively studied in chickens so far. It is also not clear if RFI-related differences in intestinal function are similar in chickens raised in different environments. The objective was to investigate differences in nutrient retention, visceral organ size, intestinal morphology, jejunal permeability and expression of genes related to barrier function, and innate immune response in chickens of diverging RFI raised at two locations (L1: Austria; L2: UK). The experimental protocol was similar and the same dietary formulation was fed at the two locations. Individual BW and feed intake (FI) of chickens (Cobb 500FF) were recorded from d 7 of life. At 5 wks of life, chickens (L1, n = 157; L2 = 192) were ranked according to their RFI and low, medium and high RFI chickens were selected (n = 9/RFI group, sex and location). RFI values were similar between locations within the same RFI group and increased by 446 and 464 g from low to high RFI in females and males, respectively. Location, but not RFI rank, affected growth, nutrient retention, size of the intestine and jejunal disaccharidase activity. Chickens from L2 had lower total body weight gain and mucosal enzyme activity but higher nutrient retention and longer intestines than chickens at L1. Parameters only determined at L1 showed increased crypt depth in the duodenum and jejunum and enhanced paracellular permeability in low versus high RFI females. Jejunal expression of *IL1B* was lower in low versus high RFI females at L2, whereas that of *TLR4* at L1 and *MCT1* at both locations was higher in low versus high RFI males. Correlation analysis between intestinal parameters and feed efficiency metrics indicated that feed conversion ratio was more correlated to intestinal size and function than was RFI. In conclusion, the rearing environment greatly affected intestinal size and function, thereby contributing to the variation in chicken RFI observed across locations.

53 Key words: chickens, intestinal permeability, gene expression, visceral organs, residual feed
54 intake

INTRODUCTION

In chicken production, traits related to production efficiency have been under selection for generations, resulting in a correlated improvement of feed efficiency (**FE**) (Zuidhof et al., 2014). Nowadays, residual feed intake (**RFI**), calculated as the difference between predicted and observed feed intake (**FI**), is often used as the metric for FE in livestock and reflects inherent inter-animal variation in biological processes associated with FE (Bottje and Carstens, 2009). By using disproportionate amounts of energy relative to their weight, the gastrointestinal tract and liver are important energy sinks, accounting for about 20% of the whole body energy expenditure (Choct, 2009). The extent of intestinal nutrient uptake is modulated by the interplay between digestive secretions and the condition of the intestinal absorptive surface (Caspary, 1992; Nain et al., 2012). Moreover, the integrity of the intestinal epithelium modifies nutrient uptake, translocation of intestinal antigens, and thus growth efficiency (Choct, 2009). Therefore, it can be assumed that, in order to maximize the utilization of dietary energy and nutrients, more feed efficient animals should have greater digestive and absorptive capacity than less efficient animals. This hypothesis has been more intensively studied in beef cattle and pigs than in meat-type chickens (Montagne et al., 2014; Fitzsimons et al., 2014; Vigors et al., 2016; Metzler-Zebeli et al., 2017a). One characteristic of low RFI animals is a drastically lower FI compared to less feed efficient (high RFI) animals (Metzler-Zebeli et al., 2016; Vigors et al., 2016). In general, FI substantially influences the size and energy requirement of the intestine and can therefore elevate the basal nutrient demands of the animal (Johnson et al., 1990; Herd and Arthur, 2009; Fitzsimons et al., 2014). Increasing the daily protein intake has been shown to decrease the efficiency of protein digestion and amino acid absorption in chickens (Hernández et al., 2012). Available data for chickens are inconclusive regarding whether the increased FI associated with high RFI chickens results in enlarged visceral organs and reduced nutrient digestion. Some authors reported no relation between RFI and nutrient digestibility (Luiting et al., 1994), whereas

81 others found positive relationships between RFI and fecal nitrogen excretion (Aggrey et al.,
82 2014). Likewise, low RFI has also been associated with lighter liver weight in pullets (van
83 Eerden et al., 2004a), whereas adult hens of low RFI had larger liver size and duodenal
84 absorptive villi surface than high RFI hens (Nain et al., 2012). In addition, evidence for RFI-
85 related variation in the function of the duodenum in meat-type chickens has been found using
86 global gene expression profiling (e.g., Aggrey et al., 2014; Lee et al., 2015), whereas similar
87 information for other intestinal segments is scarce.
88 Most evidence regarding the underlying mechanisms for variation in RFI in chickens has
89 been derived from one contemporary population of chickens of similar biotype and
90 management conditions at one experimental setting (Bottje and Carstens, 2009). Therefore, it
91 is not known whether the underlying biological differences for diverging RFI concur when
92 chickens are raised in different rearing environments. Substantial batch-to-batch variation was
93 reported for the intestinal microbiota of chickens raised in one environment (Stanley et al.,
94 2013, 2016; Ludvigsen et al., 2016). The intestinal microbiota differed in chickens of
95 diverging FE but FE-effects on the intestinal microbiota varied between batches (Stanley et
96 al., 2016). As the intestinal microbiota influence development and function of the gastro-
97 intestinal tract of chicks early in life (Schokker et al., 2015), environment-dependent
98 differences in the intestinal microbiota may affect the RFI-related intestinal function as well.
99 This led to the hypothesis that the environment may modify RFI-related differences in
100 intestinal size, structure and function in chickens which will have implications for
101 intervention approaches to manipulate underlying physiological mechanisms in attempts to
102 improve FE in chickens.
103 The objective was therefore to examine the differences in nutrient digestion, visceral organ
104 size, intestinal morphology, intestinal permeability, expression of genes in relation to barrier
105 function, and innate immune response of the jejunal mucosa in chickens of diverging RFI
106 raised at two different experimental sites under very similar experimental conditions.

MATERIALS AND METHODS

Chicken trials

Two chicken experiments using similar protocols comprising the experimental setup, diets, data and sample collection were conducted at the Institute of Animal Nutrition and Functional Plant Compounds [University of Veterinary Medicine Vienna, Austria; location 1 (**L1**)] and at the Agriculture Branch of Agri-Food and Biosciences Institute [Hillsborough, Northern Ireland, United Kingdom; location 2 (**L2**)] using a completely randomized study design. At both locations three replicate batches were performed using day-old mixed-sex Cobb 500FF chicks, resulting in a total population of 78 females and 79 males at L1 and in a total population of 96 females and 96 males at L2. Within each replicate batch, equal numbers of females and males, except for batch 2 with one more male at L1, were used. Due to the geographic distance, chickens at L1 and L2 came from different commercial hatcheries. The chicken batches were run simultaneously at both locations. All animal experimentation procedures were approved by the institutional ethics committee at the University of Veterinary Medicine Vienna and the Austrian national authority according to paragraph 26 of Law for Animal Experiments, Tierversuchsgesetz 2012 – TVG 2012 (GZ 68.205/0131—II/3b/2013). At Agri-Food and Biosciences Institute the animal procedures were conducted under Project Licence PPL 2781 obtained from the Department of Health, Social Services and Public Safety (DHSSPS) and which adhere to the Animals (Scientific Procedures) Act, 1986.

At hatch, chicks were sexed at the hatcheries and transported to L1 and L2 within the first day of life. Upon arrival, chickens were weighed and group-housed for the first days of life. From d 7 of life, chickens were individually housed until the end of the experimental period. The cage floors were made of wire mesh (10 mm × 10 mm) and padded with rubber tubing. The temperature was maintained at 33°C for the first 5 days after which it was gradually decreased until reaching a temperature of 21°C on d 21 of life. The chickens received 18

hours of light and 6 hours of dark. Each cage was equipped with one manual feeder and drinker with feed and demineralized water freely available.

Chickens were fed starter, grower and finisher diets based on corn and soybean meal from d 1 to 10, d 11 to 21, and d 22 to 42 of life, respectively (Supplementary Table 1). Diets did not contain anti-microbial growth promoters or coccidiostats. Starter, grower and finisher diets were mixed according to the same diet formulation at each location. At each location, starter, grower and finisher diets for the replicate batches came from the same batch of commercially prepared crumbles (starter diet) and pellets (3 mm; grower and finisher diets) and were stored in cool (< 15°C) and dry conditions for a duration of no longer than 6 months.

Data collection and feed efficiency measurement

The FI was determined weekly. Feed leftovers and spills were collected before recording feed intake on d 14, 21, 28, 35, 36 and 38 of life. Once a week (upon arrival, d 7, 14, 21, 28 and 35) and on the day when the RFI was determined (d 36 and d 38 of life at L1 and L2, respectively), body weight (**BW**) of all chickens was recorded. Determination of RFI and selection of low, medium and high RFI chickens were planned to occur for the experimental period between d 7 and d 38 of life. However, as chickens at L1 grew faster than chickens at L2, selection of low, medium and high RFI chickens at L1 took place two days earlier on d 36 of life in order to achieve approximately similar BW at euthanization and hence to minimize the effect of BW and body composition on parameters of interest. Chickens at L2 were weighed again and ranked according to their RFI value on d 38 of life. Data for net total FI (**TFI**), metabolic mid-weight (**MMW**) and total BW gain (**TBWG**) were used to estimate RFI values as the residuals over the test interval with a nonlinear mixed model in SAS (SAS Stat Inc., version 9.2; Cary; NC) as described in Metzler-Zebeli et al. (2016):

The MMW was calculated as:

MMW = [(BW at d 7 of life (g) + BW at d 36 or d 38 of life (g)) / 2]$^{0.75}$.

The RFI and RBG were calculated as:

RFI (g) = TFI - (a_1 + b_1 × MMW + b_2 × TBWG),

where a_1 is the intercept and b_1 and b_2 are partial regression coefficients of MMW and TBWG on TFI, respectively. In addition, RBG, residual intake over gain (RIG) and feed conversion ratio (FCR) for the test interval were calculated for the selected chickens:

RBG (g) = TBWG - (a_2 + b_3 × MMW + b_4 × TFI),

where a_2 is the intercept and b_3 and b_4 are partial regression coefficients of MMW and TFI on TBWG, respectively.

The RIG was calculated as:

RIG (g) = RBG (g) – RFI (g).

The FCR was calculated as:

FCR (g/g) = TFI (g) / TBWG (g).

Experimental design

In each replicate batch and location, separately for females and males, it was aimed to select the three chickens with the lowest RFI (high FE), the three chickens with the highest RFI (poor FE), and the three chickens with medium RFI which was close to zero, were selected. For all three replicate batches, each RFI group was represented by nine females and nine males at L1. At L2, in turn, six low RFI, eleven medium RFI and six high RFI female chickens and ten low RFI, nine medium RFI and nine high RFI male chickens were selected. Only FE data and excreta samples from the selected chickens at both locations were used for analysis. At the end of the experimental period, the selected chickens were euthanized to collect intestinal samples. The remaining chickens were removed from the experiment. TFI and TBWG were adjusted for the test interval from d 7 to 36 of life across locations.

185 ***Sampling procedures***

186 Total excreta were collected from the cage floor and the tray below the cage from 08:00 h
187 on d 34 to 08:00 h on d 36 of life and stored at -20°C. Chickens were weighed before being
188 sacrified between day 37 and 42 of life. At L1, selected chickens were euthanized with an
189 overdose of sodium pentobarbital (450 mg/kg, Release, WTD-Wirtschaftsgenossenschaft
190 Deutscher Tierärzte, Garbsen, Germany) by i.v. injection into the caudal tibial vein from d 37
191 to 42 of life, whereas at L2 selected chickens were sacrificed on d 41 and 42 of life. After
192 opening the abdominal cavity, the liver and the gastrointestinal tract were removed. The
193 weight of the liver and pancreas was recorded. Following collection of intestinal samples for
194 morphometric analysis, the intestinal segments were opened at the mesenterium, intestinal
195 digesta removed, and intestinal segments were washed in neutral-buffered saline and blotted
196 dry on paper towel. The weight of the dried empty intestinal segments was then determined.
197 Thereby, the empty weight of crop, gizzard and proventriculus were only measured at L1,
198 whereas the empty weight of duodenum, jejunum, ileum, ceca and colon, and the lengths of
199 the total intestinal tract and of the individual segments were determined at both locations. The
200 duodenum was defined as the segment from pylorus to the end of the pancreatic loop. The
201 ileum was defined as the segment between the ileo-cecal junction and the beginning of the
202 caudal mesenteric blood supply. To adjust for differences in BW among chickens, weight of
203 liver and pancreas, and the empty weight of intestinal segments, as well as total and
204 segmental gut length were expressed per kg of BW.

205

206 ***Mucosa sampling and enzyme activity measurement***

207 The mucosa was scraped off using a glass slide from the jejunum between the Meckel's
208 Diverticulum and 35 cm towards the duodenum. Mucosa samples were immediately snap-
209 frozen in liquid nitrogen and subsamples were stored at -80°C for subsequent brush border
210 enzyme activity measurements and RNA isolation. Enzyme activity measurements in jejunal

samples from both locations were performed at L1. Preparation of jejunal homogenates (20%, w/v) and mucosal enzyme activity measurements were performed essentially as previously described (Martin et al., 2013; Metzler-Zebeli et al., 2017a). All enzyme activities were expressed as micromoles of substrate hydrolyzed per minute per g protein (U/g protein).

Candidate gene expression

Candidate gene expression analysis for jejunal samples from both locations was performed at L1. Total RNA was isolated from jejunal mucosal scrapings of low and high RFI chickens as described (Metzler-Zebeli et al., 2015) using mechanical homogenization and the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA isolates were treated with DNase I (RNA Clean & Concentrator-5 Kit, Zymo Research, Irvine, USA). The quality of RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) and the subsequent determination of RNA integrity numbers (**RIN**). The majority of samples had a RIN between 9 and 10; five samples had RIN values between 4.5 and 6.7. Single stranded cDNA was synthesized from 1 µg of total RNA using the High Capacity Reverse Transcription Kit (Life Technologies Foster City, USA).

Primers were designed using the Primer Express Software version 3.0 (Life Technologies; Supplementary Table 2). If possible, primer pairs were located on different exons. Candidate genes were monocarboxylate transporter 1 (*MCT1*), intestinal alkaline phosphatase (*ALPI*), tight-junction proteins [claudin 1, (*CLDN1*), claudin 5 (*CLDN5*), zona occludens 1 (*ZO1*) and occludin (*OCLN*)], interleukin-1β (*IL1B*), tumor-necrosis-factor-α (*TNFA*), toll-like receptor 2 (*TLR2)* and 4 (*TLR4*) (Supplementary Table 2). In total, six potential housekeeping genes (**HKG**) were included. Beta-actin (*ACTB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-2 microglobulin (*B2M*) and ornithine decarboxylase antizyme1 (*OAZ1*) were chosen based on previous gene expression data of jejunal tissue from pigs (Metzler-Zebeli et al., 2015). The small nuclear

237 ribonucleoprotein D3 polypeptide (*SNRPD3*) was added as an additional HKG, as this gene is
238 uniformly expressed among a variety of human tissue types and has been shown to be suitable
239 for normalization of RT-qPCR data from other mammalian species (Eisenberg and Levanon,
240 2013; Scarlet et al., 2015). The expression stability of all six HKG was assessed using the
241 geNorm software tool (Vandesompele et al., 2002). The geometric mean of the two most
242 stably expressed genes (*GAPDH*, *SNRPD3*) was used for normalization of the target gene
243 expression levels.
244 Amplifications of target and housekeeping genes were performed on a ViiA 7 Real-time
245 PCR system (Life Technologies, Carlsbad, CA, USA). RT-qPCR was carried out in 20 µl
246 reactions including 25 ng cDNA template, 200 nM of each primer, 0.2 mM of each dNTP, 3
247 mM $MgCl_2$, 1 × buffer B2 (Solis BioDyne, Tartu, Estonia), 50 nM ROX reference dye
248 (Invitrogen, Carlsbad, USA), 0.4 × EvaGreen fluorescent dye (Biotium, Hayward, USA) and
249 1 unit of HOT FIREPol DNA polymerase (Solis BioDyne; Metzler-Zebeli et al., 2015). All
250 reactions were run in duplicate using the following temperature: 95°C for 10 min, 40 cycles of
251 95°C for 15 sec and 60°C for 1 min, followed by the generation of dissociation curves.
252 Reverse transcription controls (RT minus) were included in order to control for residual DNA
253 contamination. The fold change in the target gene, normalized to the mean of the two most
254 stably expressed HKG, was calculated relative to the expression of one high RFI females
255 from L1 using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Amplification efficiencies (E
256 = $10^{(-1/slope)-1}$) of all primer sets are provided in Supplementary Table 2 and prepared by using
257 a 5-fold serial dilution of samples.
258
259 ***Morphometric measurements (performed at L1 only)***
260 Pieces of the intestinal tube (1 cm) for morphometric measurements were collected from
261 the *Flexura duodeni*, Meckel's diverticulum, the first centimeter of the proximal ileum, and
262 proximal to the blind end of the ceca. Tube pieces were thoroughly washed in phosphate-

263 buffered saline and fixed in neutral-buffered (pH 7.0) formalin (4% vol/vol). After fixation,
264 intestinal tube samples were dehydrated in ethanol, cleared in xylene and embedded in
265 paraffin. Three discontinuous 3 to 4 μm-thick sections per intestinal site and chicken were
266 processed for evaluation. These sections were stained with hematoxylin and eosin. Slides
267 were examined on a Leica DM2000 light microscope (Leica Microsystems, Wetzlar,
268 Germany) and digital images were captured for morphometric analysis. The villus height
269 from the tip to the villus-crypt junction, villus width at one-third and two thirds of the length
270 of the villus, and the crypt depth from the base of the villus to the mucosa were measured
271 using the image analysis software ImageJ (version 1.47; National Institutes of Health,
272 Maryland, USA). For each trait, 15 measurements were taken from intact well-oriented, crypt-
273 villus units. The criterion for villus selection was based on the presence of intact lamina
274 propria. Villus height and width were measured at 4-times objective magnification and crypt
275 depth at 10-times objective magnification. The villus surface area was estimated (Nain et al.,
276 2012; Sohail et al., 2012):
277 Villus surface area = $2\pi \times$ (average villus width/2) $\times$ villus height.
278 In addition, the thickness of the circular and longitudinal muscular layers was measured.
279 Goblet cells were counted per 250 μm of villus or crypt epithelium profile length for a total of
280 15 replicates per gut section at a 10-times objective magnification. Intraepithelial
281 lymphocytes were counted per 400 μm villus epithelium profile length for 12 replicates per
282 intestinal section at a 20-times objective magnification.
283 As more RFI-related histo-morphological differences were observed in females, the
284 relative absorptive and secretory surface area of the jejunum in low and high RFI females was
285 additionally assessed using vertical uniform random sampling according to Howard and Reed
286 (2005). Of the paraffin-embedded samples, one-hundred 4 μm thick serial sections per tissue
287 block were cut. Every tenth section was stained with Periodic acid-Schiff reagent. The
288 distance between the examined sections was 40 μm allowing evaluation of a total length of

400 µm per jejunum sample. Pictures of the sections were taken at 4-times objective magnification and analyzed using the digital software package ELLIPSE (version 2.0.8.1, Kosice, Slovakia). Mucosa and serosa surfaces within the samples were estimated stereologically according to Howard and Reed (2005). Results are presented as mucosal surface to serosal surface ratio.

Ussing Chamber experiment (performed at L1 only)

Differences in intestinal electrophysiological parameters and permeability marker flux were evaluated for four replicate samples per chicken and three chickens per sampling day (one low, medium and high RFI chicken of the same sex) as described in Metzler-Zebeli et al. (2017b). This resulted in six observations per RFI group and sex. A 20-cm tissue tube piece for the Ussing chamber experiment was collected distal to the Meckel's diverticulum in the direction of the ileum, immediately transferred into ice-cold transport buffer (for buffer composition see Supplementary Material), which was pre-gassed with carbogen gas (95% O_2–5% CO_2), and transported to the laboratory within 10 min of the death of the animal. For each chicken, jejunal tube pieces were opened at the mesenterium and rinsed with transport buffer to remove digesta particles. Clean tissue pieces were stripped of the outer serosal layers (*Tunica serosa* and the *Tunica muscularis*). The first centimeter of the tissue sample was discarded after which four jejunal mucosal pieces were consecutively cut from the proximal 10 cm of the jejunal tube which were each mounted in an Ussing chamber. The apical and basolateral sides of the tissue piece had an exposed area of 0.91 cm^2 and were each incubated in a total volume of 10 mL serosal and mucosal buffer solution (pH 7.4, 38°C; for buffer composition, see Supplementary Material). Continuous gassing with carbogen was provided on both the mucosal and the serosal sides to ensure oxygenation and circulation of the buffer by gas lift. The temperature was maintained at 38 °C using a circulating thermostated water jacket.

Each Ussing chamber apparatus was connected to two pairs of dual channel current and voltage Ag–AgCl electrodes, which were connected via 3% agar bridges filled with 3 M potassium chloride to allow for electrophysiological measurement by a microprocessor-based voltage-clamp device and software (version 9.10; Mussler, Microclamp, Aachen, Germany). The tissue was alternatively pulsed with a positive or negative pulse of 20 µA and 100 ms duration. After an equilibration period of 20 min under open-circuit conditions, the tissue was short-circuited by clamping the voltage to zero. The potential difference (mV), short-circuit current (I_{sc}, µA/cm^2) and transepithelial resistance (Ω × cm^2) were continuously recorded using a microprocessor-based voltage-clamp device and software (version 9.10; Mussler, Microclamp, Aachen, Germany). The tissue conductance (G_T, mS/cm^2) was calculated as the reciprocal of the R_T.

After recording electrophysiological measurements for 5 min, fluorescein 5(6)-isothiocyanate (FITC; 389.38 g/mol; Sigma-Aldrich, Schnelldorf, Austria) and horse-radish peroxidase (HRP; 44,000 g/mol; Carl Roth GmbH+Co.KG, Karlsruhe, Germany) were added to final concentrations of 0.1 mM and 1.8 µM, respectively, to assess the mucosal-to-serosal flux and, as a result, the paracellular permeability of the distal jejunum. Samples from the basolateral buffer solution were taken at 60, 120 and 180 min, whereas samples from the mucosal side were collected at 70 and 170 min after marker addition to measure marker flux rates. The buffer samples were stored at −20 °C and were later analyzed for concentrations of FITC and HRP. At the end of the experiment (185 min after initiating voltage clamp), the tissue survival was monitored by adding theophylline (inhibitor of the phosphodiesterase; final concentration, 8 mmol/L) to both chamber halves. Concentrations of FITC and HRP in mucosal and serosal buffers were analyzed, and mucosal-to-serosal flux rates of FITC and HRP were calculated as described in Metzler-Zebeli et al. (2017b).

Chemical analysis of feed and feces

341 The DM content of feed and freshly dropped excreta samples was determined by oven-
342 drying at 105°C overnight (method 3.1; Naumann and Basler, 2012). Total excreta samples
343 were pooled for each chicken and freeze-dried prior to proximate nutrient analysis. Dried
344 fecal samples were ground (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany)
345 to pass through a 0.5-mm screen. Proximate nutrients [DM, crude ash, CP (nitrogen × 6.25)
346 by the Kjeldahl method] in diets and feces at both locations, and only at L1 real protein by the
347 Barnstein method, ether extract, crude fiber, total starch, sugar, calcium in diets and
348 phosphorus (P) were analyzed in diets and feces according to Naumann and Basler (2012).
349 Acid-insoluble ash (**AIA**; used as indigestible marker) contents of finisher diet and fecal
350 samples were analyzed at both locations after ashing the samples and boiling the ash with 4 M
351 hydrochloric acid (Naumann and Basler, 2012).
352 The apparent total tract digestibility (**ATTD**, %) of DM and real protein as well as the
353 apparent retention of ash, CP and P (% of intake) were calculated as:
354 ATTD or retention (%) = 100 - [100 × (% AIA in feed/% AIA in feces) × (% nutrient in
355 feces/% nutrient in feed)] [5].
356 Daily nutrient excretion was calculated as [6]:
357 Nutrient excretion (g/day) = Nutrient content in feces (g/kg) × DM intake (g/day) × (%
358 AIA in feed/% AIA in feces) [6].
359 Dry matter intake was calculated for the collection days of total excreta on d 34 and 35 of
360 life.
361
362 ***Statistical analysis***
363 Data for BW, RFI, size of intestine, liver and pancreas, jejunal permeability, brush
364 border enzyme activity, histo-morphology and candidate gene expression were first analyzed
365 for normality using Shapiro-Wilk test with the PROC UNIVARIATE in SAS (version 9.4;
366 SAS Inst. Inc., Cary, NC). The Cook's distance (Cook's D) test in SAS was used to determine

any influential observation on the model. All variables were normally distributed and analyzed by ANOVA using the PROC MIXED procedure in SAS. Overall, two different models were run. The first accounted for the fixed effects of sex, batch, location and RFI group. Because chickens were sacrificed at different days of life and to consider that chickens were consecutively sampled, the first model included the random effects of chicken nested within day of life × chicken order at sacrifice. Sex as fixed effect was significant for most parameters; therefore, variables were analyzed separately for female and male chickens using the second model. This model was fitted to take into account the fixed effects of RFI group, experimental location and their two-way-interaction. The random effect considered the chicken nested within batch, day of life × chicken order at slaughter. For parameters only determined at L1 (i.e., jejunal permeability, morphology and weight of crop, gizzard and proventriculus), location and the RFI × location interaction were omitted as fixed effects. Where applicable, orthogonal contrasts were used to evaluate linear effects of RFI group. Degrees of freedom were approximated by the method of Kenward-Roger. Least squares means were computed using the pdiff statement and significance declared at $P \leq 0.05$. A trend was considered at $0.05 < P \leq 0.10$.

For variables that were available from both locations, Pearson's correlation analysis (PROC CORR in SAS) was used to establish and quantify the relationship between individual RFI, RBG, RIG and FCR, TFI and TBWG values and the intestinal variables visceral organ size, nutrient retention and excretion, and mucosal enzyme activity and gene expression in the jejunum.

RESULTS

Chicken performance and feed efficiency

The RFI ranged on average from -231 to 215 g for low to high RFI in females and from -197 to 267 g for low to high RFI in males ($P < 0.001$; Supplementary Table 3) representing a

393 difference of 330 and 498 g in TFI between the most and least efficient female and male
394 chickens ($P < 0.001$) respectively. Body weight development and TBWG were similar among
395 chickens of diverging RFI. The FCR linearly increased on average by 13% from low to high
396 RFI in both sexes ($P < 0.001$). Total feed intake was similar across locations, whereas female
397 and male chickens gained 354 and 418 g less, respectively, at L2 compared to L1 ($P < 0.001$).
398 This led to a 12 % lower FCR in chickens at L1 compared to L2 ($P < 0.001$). At sacrifice,
399 male chickens at both locations had similar BW across locations and RFI group, whereas
400 female chickens at sacrifice weighed 270 g more at L1 than at L2 ($P = 0.001$), but their BW
401 was similar among RFI groups.
402
403 ***Nutrient retention and excretion***
404 Irrespective of sex, selected chickens at L2 had higher nutrient retention and lower nutrient
405 excretion than at L1 ($P < 0.01$; Table 1). Female chickens at L2 but not at L1 had increased
406 ATTD of DM from low to high RFI, whereas male chickens at L1 had a 20%-higher daily
407 DM excretion from low to high RFI ($P < 0.05$). In males, retention of CP linearly decreased
408 from low to high RFI, while daily CP excretion increased from low to high RFI in male and
409 female chickens at L1 ($P < 0.05$). Both males and females at L1 had a linearly increasing
410 ATTD and decreasing daily excretion of real protein from low to high RFI ($P < 0.05$),
411 whereas uric acid-nitrogen excretion was similar for the RFI groups.
412
413 ***Visceral organ weights and gut length***
414 The size and weight of the intestinal tract of chickens was largely affected by location ($P <$
415 0.05; Table 2) and hence RFI-related differences in visceral organ size were different between
416 locations. In particular, the length and weight of the ileum were 3.5- and 5-times greater at L2
417 compared to L1 ($P < 0.001$), irrespective of sex. Overall, FE × location interactions were
418 observed for liver and ileum weight in females, as well as for ceca length and colon weight in

males which indicated FE-related differences at L2 but not at L1 ($P < 0.05$). Accordingly, at L2, low RFI female chickens had a heavier liver and ileum compared to medium RFI females ($P < 0.05$). Also at L2, male chickens of low RFI had shorter ceca compared to high RFI males ($P < 0.05$), whereas medium RFI males had a heavier colon than low and high RFI males ($P < 0.05$). Moreover, in males, the weight of the liver increased by 21% from low to high RFI at L1 ($P < 0.05$) but not at L2.

Intestinal morphology

Intestinal histo-mophological measures were only determined in chickens of L1. Differences in intestinal morphology due to diverging RFI were mostly found in female chickens, whereas in males differences could not be distinguished (Table 3; Supplemental Table 4). As such, crypt depth linearly increased from low to high RFI in the duodenum of females ($P < 0.05$; Table 3). Likewise, jejunal villus height and crypt depth linearly increased from low to high RFI in the mid-jejunum of females ($P < 0.05$), leading to a 29%-increase in jejunal apparent villus surface area from low to high RFI ($P < 0.05$). Because of these differences for female chickens, we also determined the relative absorptive and secretory surface area in the jejunum of chickens; however, this was similar between low RFI and high RFI females. Goblet cell and intraepithelial lymphocyte numbers were not different between the RFI groups at any intestinal segment.

Mucosal enzyme activity

Location affected the mucosal activity of maltase and sucrase in the mid-jejunum of males and females by being ~10 to 50% higher in chickens at L1 than at L2 ($P < 0.01$; Table 4), whereas the detectable lactase activity was similar at both locations. Variation in jejunal maltase activity between the RFI groups was only observed in males at L1 where lower activity was found in low RFI compared to medium RFI animals ($P < 0.05$).

445

446 ***Candidate gene expression in jejunal mucosa***

447 Location-related differences in the relative expression of genes in the mid-jejunal mucosa
448 were observed for expression of *OCLN* and *IL1B* in both sexes and for the expression of *ZO1*,
449 *TNFA*, *TLR2* and *IAP* in males which were higher at L2 than at L1 ($P < 0.05$; Table 5). Only
450 the relative *MCT1* expression of the jejunal mucosa was about 22% higher at L1 compared to
451 L2 ($P < 0.05$). A RFI-related variation in relative gene expression was distinguishable for the
452 expression of *IL1B* in females, and of *TLR4* and *MCT1* in males ($P < 0.05$). Low RFI females
453 had a lower relative *IL1B* expression than high RFI chickens at L2 but not at L1 ($P < 0.05$). In
454 contrast, relative expression of *TLR4* in the jejunum was 50% higher in low RFI males
455 compared to high RFI males at L2 ($P < 0.05$) but not at L1. Likewise, relative expression of
456 the short-chain fatty acid transporter *MCT1* was higher in low RFI males compared to high
457 RFI males and this effect was observed at both locations ($P < 0.001$).

458

459 ***Jejunal permeability***

460 Short-circuit current, G_T and mucosal-to-serosal flux rates of FITC and HRP were used to
461 determine electrogenic ion transport and tissue permeability in the distal jejunum (Table 6).
462 Gut electrophysiological parameters (I_{sc} and G_T) and marker fluxes were not different in the
463 distal jejunum of male chickens of diverging RFI, whereas RFI-related differences were
464 detectable in females. Females had a linearly decreasing I_{sc} ($P < 0.05$) and G_T ($P < 0.001$)
465 from low to high RFI in the distal jejunum. Similarly, the mucosal-to-serosal flux of FITC
466 linearly decreased ($P < 0.05$) from low to high RFI in the distal jejunum of females.

467

468 ***Pearson's correlations among RFI and intestinal size and function***

469 Pearson's correlation analysis was used to correlate FE metrics, TFI and TBWG data with
470 variables that were measured at both locations (Supplemental Table 5 and 6). Only few

correlations between chicken's TFI and intestinal variables, such as jejunal relative *TLR4* and *MCT1* expression in males, could be established. Irrespective of sex, chicken's TBWG correlated ($r \geq 0.30$; $P < 0.05$) with small and large intestinal length and weight as well as with retention and excretion of DM and CP. It was also correlated with jejunal expression of *IL1B, TLR2* (negatively) and *TNFA* (positively) in females ($P < 0.05$). Positive correlations between RFI and excretion of DM and CP could be established in males but not in females. In females, jejunal expression of *IL1B* positively correlated to chicken's RFI ($r = 0.40$; $P < 0.05$). Other variables, such as visceral organ size and mucosal enzyme activity in the jejunum, were not correlated to chicken's RFI value. Correlations between RIG and intestinal variables were similar to those of RFI, whereas chicken's RBG correlated with duodenal weight in females as well as excretion of DM and CP in males ($r \geq 0.33$; $P < 0.05$; Supplementary Tables 5 and 6). Many variables including length and weight of almost all segments of the small and large intestines in both sexes, retention and excretion of DM and CP in both sexes, jejunal maltase and sucrase activity in females and the relative expression of *IL1B* and *ZO1* (females) as well as of *OCLN*, *CLDN1* and *MCT1* (males) correlated to chicken's FCR ($r \geq 0.33$; $P < 0.05$; Supplementary Tables 5 and 6).

Moreover, nutrient retention and intestinal length and weight were correlated and showed weak ($r = 0.30$-0.49; $P < 0.05$), medium ($r = 0.50$-0.69; $P < 0.001$) or strong ($r = 0.70$-0.87; $P < 0.001$) relationships for almost all combinations in both sexes (Supplemental Table 7).

DISCUSSION

In the current study all birds represented Cobb 500FF genetics (Supplementary Figures 1) and the trials across the two geographical locations were designed to mirror each other as closely as possible. However, the present results demonstrate that the environment has a great impact on RFI-related differences in intestinal structure and function in chickens. Strong

location-associated effects existed for all parameter categories including growth performance, nutrient retention and excretion, intestinal size, mucosal disaccharidase activity and expression of some target genes in the jejunum. Only a few intestinal variables, such as the excretion of DM and CP in males as well as jejunal expression of *IL1B* in females, were clearly associated with chicken's RFI at both locations and as such might be targeted to improve RFI in chickens, irrespective of the environment in which the chickens were raised. Present results from L1 further emphasized that biological profiles of RFI-related variation in intestinal size, structure and function should be separately generated for males and females as differences in small intestinal structure and jejunal permeability was associated with variation in RFI in females but not in males. Regardless of the poor correlation between RFI and intestinal variables, FCR in chickens appeared to be more useful for the identification of common intestinal features that explain FE variation across different environments.

Due to the similar experimental procedures, the largely diverging TBWG from d 7 to 36 of life across locations was not expected but was likely associated with the longer and heavier small and large intestines found in chickens at L2 compared to L1. The intestinal tract has a high metabolic activity; therefore, changes in its size can have a profound impact on the energy efficiency and growth of the whole animal (Johnson et al., 1990; Choct et al., 1999; Herd and Arthur, 2009). The higher nutrient retention in chickens at L2 compared to chickens at L1 was also not expected and was evidently not sufficient to compensate for the higher basal needs of the intestine to maintain growth. Due to the higher retention of nitrogen, chickens from L2 had a reduced daily excretion of environmental pollutants compared to chickens from L1. However, at production scale, lower growth performance adds days to reach market weight, which may counterbalance a reduced daily excretion of nitrogen (Willems et al., 2013). The reduced mucosal maltase and sucrase activity in the mid-jejunum of chickens from L2 compared to chickens at L1 may be also seen as an adaptation to the

longer small intestine. Mucosal lactase activity was equal across locations and might represent similar microbial disaccharidase activity.

To explain the diverging intestinal development and function in chickens from the two locations, a genetic influence is probable, although chicks were not related to each other (Supplementary Figure 2 and Supplementary Table 8). A certain variation probably originated from the diets. As the diets were produced locally, natural variation in nutrient concentrations of the raw materials used to prepare the diets at the two locations, i.e. corn and soybean meal (e.g., dietary fiber composition; Rodehutscord et al., 2016), may have altered digestive, absorptive and fermentative processes, thereby leading to the diverging intestinal size and performance. Notably, the protein content of the starter diet at L1 was 2 % higher than at L2 which may have had consequences for intestinal development, muscle protein metabolism and growth (Everaert et al., 2010). With regards to intestinal development, nutrient digestion and growth, the microbial colonization is another important factor to consider which is also influenced by the diet (Oakley et al., 2014; Apajalahti and Vienola, 2016). The early microbial colonization largely influences the gene expression profiles in the jejunal mucosa of chickens within the first weeks of life (Schokker et al., 2015). Microbes encountered in the environment (e.g., personnel, housing, water and diet) probably differed between locations, modifying the early microbial colonization and successional changes during the test period. Location-related differences in the relative expression of genes related to the innate immune response in both sexes and expression of the short-chain fatty acid transporter *MCT1* at the jejunal mucosa in males also point to diverging microbial profiles and activity between locations.

In accordance with earlier observations in laying hens and broilers (Luiting et al., 1994), as well as in other livestock species such as beef cattle and pigs (Fitzsimons et al., 2014; Montagne et al., 2014), the present findings supported that the digestion of nutrients played a small role in the variation of RFI in our chicken populations across both locations. Only at L1,

547 selection of chickens for RFI reduced the excretion of nitrogenous substances which was
548 mainly caused by a reduction in nitrogen losses from the digestive tract as indicated by the
549 excretion of real protein and uric acid.
550 Increasing the FI level may enlarge the intestinal size to metabolize the supplied nutrients
551 which subsequently increases the intestinal energy demand (Johnston et al., 1990). Previous
552 studies in laying hens and chickens either selected for RFI or AME_N, respectively, supported
553 heavier intestinal tracts in less efficient animals (de Verdal et al., 2010; Van Eerden et al.,
554 2004a). In spite of the current differences in TFI, visceral organ size was similar among RFI
555 groups across locations. Only by analyzing the data individually for location and sex, RFI-
556 related linear patterns in visceral organ size became apparent. As such, the weight of the ceca
557 may have contributed to the RFI-variation in females at L2. At L1, liver weight may explain
558 some of the variation in RFI of male chickens, thereby supporting the theory of greater basal
559 energy needs in less efficient animals (van Eerden et al., 2004a; Fitzsimons et al., 2014).
560 Besides visceral organ size, we observed strong sex-related differences in RFI-effects on
561 intestinal structure and functioning at L1. Notably, in females, but not in males, differences in
562 small intestinal structure (e.g., villus height in jejunum and crypt depth in duodenum and
563 jejunum) and jejunal permeability contributed to the variation in RFI. In general, longer villi
564 may be expected in more efficient chickens, allowing for greater absorption of nutrients and
565 hence compensating lower FI (Caspary et al., 1992; de Verdal et al., 2010). In contrast to this
566 proposition and to findings in laying hens (Nain et al., 2012), but corresponding to
567 observations in chickens (de Verdal et al., 2010), our results for smaller jejunal villus height
568 and apparent villus surface area in low RFI females supports an energy saving mechanism by
569 reducing the maintenance needs for the renewal of the epithelial surface in the jejunum. To
570 confirm this and similar to de Verdal et al. (2010), smaller crypts were found in the duodenum
571 and jejunum of more feed efficient females as longer villi have deeper crypts to ensure their
572 renewal (Samanya and Yamauchi, 2002; de Verdal et al., 2010). Since the maltase and

573 sucrase activities per gram of mucosal protein at the jejunal brush border were similar among
574 females of diverging RFI, it can be speculated that the greater villus surface in high RFI
575 females augmented glucose release at the jejunal brush border.

576 Unlike pigs selectively bred for low and high RFI (Mani et al., 2013), the present jejunal
577 electrophysiological data and mucosal-to-serosal flux of FITC in females indicated that part
578 of the compensation of the reduced FI to maintain similar growth may be explained by greater
579 paracellular nutrient uptake in the distal jejunum. In spite of this, gene expression levels of
580 *ZO1*, *OCLN*, *CLDN1* and *CLDN5* in mid-jejunal mucosa scrapings were not different among
581 female chickens of diverging RFI, neither at L1 nor at L2. All four genes code for proteins
582 that are important components of the tight junction protein complex which establishes the
583 paracellular barrier (Turner, 2009). Due to translational regulation of gene expression, levels
584 of tight junction proteins likely did not correspond to the functional protein level. However,
585 correlation analysis indicated that a certain association between chicken's FE and up-
586 regulated expression of tight junction protein genes existed when FCR was used as a FE
587 metric. It should also be considered when comparing the results of the gene expression and
588 Ussing chamber experiments that tissue samples did not originate from the same jejunal spot
589 and the regulation of tight junction proteins and paracellular permeability may have differed
590 between the mid and distal jejunum.

591 Greater energy fueling of the basal immune response may have a negative impact on feed
592 efficiency (Mani et al., 2013). Previously, differences in the contribution of the immune
593 system to variation in RFI were more often reported in chickens under challenge conditions
594 (Cotter and Van Eerden, 2006). As part of the innate defense mechanism, similar goblet cell
595 numbers between chickens of diverging RFI may imply similar mucin production. Likewise,
596 intraepithelial lymphocytes along the small intestine were equal among RFI groups at L1 and
597 RFI-related differences in the expression of genes related to the innate immune response were
598 small. Lower *IL1B* expression in the jejunum of low RFI females was in line with the lower

599 cytokine expression in the colon of low RFI pigs which may indicate a potential energy
600 saving mechanism compared to high RFI animals (Vigors et al., 2016). In contrast, low RFI
601 male birds in the present study had a greater *TLR4* expression in the jejunal mucosa. Toll-like
602 receptor 4 interacts with lipopolysaccharide on the cell wall of gram-negative bacteria
603 (Tremaroli and Bäckhed, 2012) and may therefore indicate differences in the bacterial
604 composition of the jejunum towards a greater proportion of gram-negative bacteria in low RFI
605 birds. Along with this, by being substrate induced (Cuff et al., 2002), the increased jejunal
606 *MCT1* expression in low RFI males may be associated with a greater generation of short-
607 chain fatty acids.
608 In the correlation analysis, data from both locations were used and results support a weak
609 relationship between RFI in chickens and intestinal size, structure and functioning. Evidence
610 for the contribution to the variation of RFI of chickens could only be established for excretion
611 of DM and CP in males. Controversially, many intestinal parameters correlated to the TBWG
612 and FCR of the selected chickens. Chicken's FCR values corresponded to chicken's RFI
613 value but they were higher at L2 than at L1. Hence, by taking the differences in TBWG into
614 account, FCR may better reflect location-associated differences in intestinal variables. Based
615 on FCR, some variation in the FE of female chickens can be explained by increased nutrient
616 retention, lower expression of genes related to the innate immune response (*IL1B* and *ZO1*),
617 higher disaccharidase activity at the jejunal mucosa and smaller intestinal size in low FCR
618 animals. In males, underlying mechanisms for improved FCR at intestinal level could be
619 nutrient retention, smaller intestinal size, up-regulated expression of *OCLN* and lower
620 expression of *CLDN1* and *MCT1*.
621 In conclusion, aside from sex-related variation, the present results showed that the
622 environment in which the chickens were raised largely affected differences in intestinal
623 structure and function which in turn contributed to the variation in RFI of the selected
624 chickens. Location-related variation in intestinal size, nutrient digestion and jejunal gene

625 expression may have been associated with the different growth rate of chickens between L1
626 and L2. At L1, energy saving mechanisms, such as shorter villi and crypts, increased
627 paracellular permeability and improved protein retention in females and reduced liver size in
628 males contributed to improved RFI. At L2, low RFI was mainly associated with lower ATTD
629 of DM and heavier ilea in females and higher jejunal *TLR4* expression in male chickens. By
630 contrast, according to the correlation analysis, lower excretion of DM and CP in males and
631 jejunal *IL1B* expression in females were common factors reflecting a lower RFI at both
632 locations. Due to the greater RFI-associated variation in gut structure and function in females
633 than in males, this may imply that the RFI may be more applicable for female chickens than
634 for males in selection programs. Controversially, present correlations also suggested that the
635 FCR may better reflect intestinal function profiles linked to FE in chickens raised in different
636 environments.
637

638 **ACKNOWLEDGEMENTS**

639 This project (ECO-FCE) has received funding from the European Union's Seventh
640 Framework Programme for research, technological development and demonstration. The
641 authors would like to thank G. Kvapil, S. Sharma, A. Dockner, M. Wild, S. Leiner, A.
642 Flemming and B. Machac ((University of Veterinary Medicine Vienna) for laboratory
643 assistance. The technical staff at the Agri-Food and Biosciences Institute are also gratefully
644 thanked for their care of the animals and expertise when conducting the experiment.
645

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792 **Table 1.** Least squares means of nutrient digestibility, retention and excretion in female and male broiler chickens of diverging residual feed intake

793 (RFI) raised at two different locations

	Location 1				Location 2						
Item[1]	Low RFI	Medium RFI	High RFI	SEM	Low RFI	Medium RFI	High RFI	SEM	FE, $P^{2,3}$	location, P^{2}	FE × location, P^{2}
Females											
Dry matter											
ATTD (%)	73.2	73.1	74.2	1.32	78.2	80.9	82.8	1.39	0.15	<0.001	0.43
Excretion (g/day)	44.4	47.5	46.8	2.21	34.4	31.8	30.1	2.32	0.85	<0.001	0.30
Crude protein											
Retention (%)	57.7	52.2	52.7	2.50	70.6	76.4	77.8	2.63	0.89	<0.001	0.051
Excretion (g/day)	13.6^{b}	16.4^{ab}	16.7^{a}	0.92	7.0	7.2	6.9	0.95	0.22	<0.001	0.23
ATTD of real protein (%)[4]	68.2^{a}	64.1^{ab}	63.9^{b}	1.45	-	-	-	-	0.086*	-	-
Real protein excretion (g/d)[4]	11.4^{b}	13.7^{ab}	14.2^{a}	0.86	-	-	-	-	0.065*	-	-
Uric acid-nitrogen excretion (g/d)	0.35	0.43	0.40	0.05	-	-	-	-	0.56	-	-
Crude ash											
Retention (%)	20.2	20.5	21.4	5.07	40.0	35.2	46.0	5.32	0.51	<0.001	0.62
Excretion (g/day)	6.9	7.4	8.1	0.55	9.3	8.3	7.4	0.58	0.86	0.007	0.27
Phosphorus											
Retention (%)	57.9	55.3	55.3	2.06	-	-	-	-	0.59	-	-
Excretion (g/day)	0.40	0.47	0.48	0.04	-	-	-	-	0.21	-	-
Males											
Dry matter											
ATTD (%)	74.4	73.2	71.1	1.73	77.5	80.8	81.8	1.73	0.83	<0.001	0.093
Excretion (g/day)	48.4	51.6	58.8	3.42	36.9	36.8	41.3	3.42	0.081*	<0.001	0.68
Crude protein											

Retention (%)	61.7	57.4	52.7	1.71	72.2	76.3	76.4	2.72	0.63	<0.001	0.053
Excretion (g/day)	14.0	15.9	18.6	1.16	8.3	8.4	9.2	1.17	0.062	<0.001	0.28
ATTD of real protein (% of intake) [4]	70.7[a]	67.6[ab]	63.4[b]	2.25	-	-	-	-	0.090*	-	-
Real protein excretion (g/d) [4]	12.0[b]	13.5[ab]	16.2[a]	1.2	-	-	-	-	0.061*	-	-
Uric acid-nitrogen excretion (g/d)	0.38	0.40	0.32	0.05	-	-	-	-	0.54	-	-
Crude ash											
Retention (%)	26.5	23.1	21.9	5.85	31.6	37.1	41.9	5.86	0.88	0.009	0.43
Excretion (g/day)	7.3	11.1	10.0	0.68	9.4	7.8	8.4	0.69	0.12	<0.001	0.76
Phosphorus											
Retention (%)	61.5	56.5	54.0	2.93	-	-	-	-	0.21	-	-
Excretion (g/day)	0.43	0.49	0.55	0.04	-	-	-	-	0.16	-	-

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; ATTD, apparent total tract digestibility; FE, feed efficiency; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2] *P*: probability level of fixed effects feed efficiency, location and their two-way interaction.

[3]Linear polynominal contrast: $*P \leq 0.05$.

[4]Nitrogen × 6.25.

800 **Table 2.** Least squares means of visceral organ weight and intestinal length in female and male broiler chickens of diverging residual feed intake

801 raised at two different locations

	Location 1				Location 2						
Item[1]	Low RFI	Medium RFI	High RFI	SEM	Low RFI	Medium RFI	High RFI	SEM	FE, P^2	location, P^2	FE × location, P^2
Females											
Organ weight (g/kg BW)											
Liver	20.16	22.90	23.36	1.122	24.17[a]	20.12[b]	22.30[ab]	1.193	0.50	0.96	0.019
Pancreas	1.60	1.82	1.72	0.087	1.77	1.63	1.76	0.093	0.81	0.96	0.13
Crop	2.42	2.33	2.35	0.211	-	-	-	-	0.76	-	-
Gizzard	3.04	3.56	3.72	0.355	-	-	-	-	0.38	-	-
Proventriculus	7.62	7.85	7.87	0.368	-	-	-	-	0.87	-	-
Duodenum	4.21	4.73	4.60	0.277	5.47	5.15	4.92	0.294	0.79	0.007	0.23
Jejunum	12.26	12.36	13.27	0.879	10.58	8.87	9.45	0.934	0.60	<0.001	0.48
Ileum	1.48	1.44	1.60	0.234	8.08[a]	6.30[b]	7.36[ab]	0.249	0.001	<0.001	0.003
Average cecum[3]	0.72	0.66	0.70	0.118	2.73	2.45	2.29	0.126	0.18	<0.001	0.26
Colon	0.76	0.89	0.84	0.091	1.15	1.01	1.14	0.096	0.91	0.001	0.34
Length (cm/kg BW)											
Duodenum	9.99	10.53	10.23	0.534	11.35	11.19	11.56	0.555	0.91	0.015	0.75
Jejunum	27.98	31.58	30.22	1.271	28.55	25.99	28.75	1.352	0.67	0.050	0.063
Ileum	8.31	7.84	8.86	1.015	28.33	27.15	29.88	1.079	0.19	<0.001	0.70
Average cecum[3]	5.02	5.00	5.18	0.273	7.05	7.05	6.71	0.290	0.94	<0.001	0.58
Colon	2.46	2.59	2.59	0.170	3.05	2.80	3.10	0.199	0.72	0.008	0.57
Males											
Organ weight (g/kg BW)											

Liver	19.47	19.50	23.43	1.003	21.08	20.42	22.16	0.986	0.012	0.61	0.33
Pancreas	1.64	1.61	1.38	0.099	1.44	1.59	1.54	0.097	0.37	0.80	0.17
Crop	2.74	3.46	3.34	0.328	-	-	-	-	0.28	-	-
Gizzard	3.40	3.15	3.39	0.304	-	-	-	-	0.80	-	-
Proventriculus	8.10	7.66	7.85	0.502	-	-	-	-	0.83	-	-
Duodenum	3.94	4.39	4.84	0.324	4.95	5.32	5.36	0.319	0.13	0.003	0.71
Jejunum	11.77	14.02	13.53	0.997	9.52	9.77	9.76	0.980	0.41	<0.001	0.57
Ileum	1.46	1.54	1.6	0.346	7.33	7.86	7.66	0.340	0.64	<0.001	0.82
Average cecum[3]	0.68	0.66	0.67	0.120	2.67	2.71	2.46	0.118	0.53	<0.001	0.51
Colon	0.66	0.66	0.74	0.063	0.90[b]	1.21[a]	1.00[b]	0.062	0.049	<0.001	0.025
Length (cm/kg BW)											
Duodenum	9.40	9.34	9.89	0.488	9.94	11.66	9.93	0.480	0.22	0.018	0.060
Jejunum	28.07	28.76	28.71	1.571	23.12	25.00	24.17	1.545	0.70	0.001	0.93
Ileum	7.52	6.78	7.98	0.706	25.59	27.16	25.28	0.670	0.82	<0.001	0.086
Average cecum[3]	4.92	4.69	4.73	0.228	5.77[b]	6.73[a]	6.06[b]	0.224	0.23	<0.001	0.036
Colon	2.08	2.09	2.24	0.124	2.53	2.83	2.69	0.122	0.33	<0.001	0.39

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; BW, body weight; FE, feed efficiency; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2]*P*: probability level of fixed effects feed efficiency, location and their two-way interaction.

[3]Average cecum represents the average (weight or length) of both ceca.

808 **Table 3.** Least squares means of morphological characteristics of different intestinal segments

809 in female broiler chickens of diverging residual feed intake raised at location 1

Item[1]	Low RFI	Medium RFI	High RFI	SEM	FE, $P^{2,3}$
Duodenum					
Villus height (µm)	1375	1579	1547	177.1	0.12
Villus width (µm)	178	180	164	6.3	0.15
Crypt depth (µm)	131	141	159	6.1	0.010*
Muscle layer (µm)	183	218	211	13.4	0.18
Villus height:crypt depth-ratio	10.6	11.3	9.8	0.49	0.13
Apparent villus surface area (mm^2)	0.68	0.83	0.83	0.072	0.29
Goblet cells per 250 µm villus surface	8.7	8.5	8.6	0.61	0.98
Intraepithelial lymphocytes per 400 µm villus surface	10.4	10.1	10.0	0.65	0.89
Jejunum					
Villus height (µm)	893	920	1048	50.7	0.080*
Villus width (µm)	129	153	149	7.5	0.080
Crypt depth (µm)	95	102	113	5.0	0.036*
Muscle layer (µm)	179	189	195	15.8	0.79
Villus height:crypt depth-ratio	9.4	9.1	9.4	0.41	0.83
Apparent villus surface area (mm^2)	0.38	0.43	0.49	0.036	0.117*
RASS (mucosal surface, µm/serosal surface, µm)[4]	17.6	-	16.6	1.11	0.54
Goblet cells per 250 µm villus surface	11.0	11.8	11.1	0.52	0.79
Intraepithelial lymphocytes per 400 µm villus surface	13.2	12.4	11.6	0.74	0.34
Ileum					
Villus height (µm)	624	647	741	42.1	0.14
Villus width (µm)	138	152	150	9.5	0.55
Crypt depth (µm)	98	98	108	4.7	0.22
Muscle layer (µm)	229	224	236	17.2	0.88
Villus height:crypt depth-ratio	6.3	6.7	6.9	0.37	0.53
Apparent villus surface area (mm^2)	0.28	0.31	0.35	0.069	0.38
Goblet cells per 250 µm villus surface	16.5	16.5	15.2	1.11	0.64
Intraepithelial lymphocytes per 400 µm villus surface	12.3	12.1	12.2	0.93	0.99
Ceca					
Crypt depth (µm)	320	317	331	24.6	0.91
Goblet cells per 250 µm villus surface	6.5	6.6	6.3	0.61	0.92

810 Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); FE, feed efficiency; RFI, residual feed

811 intake.

812 [1]Values are least squares means ± standard error of the mean (SEM).

[2]*P*: probability level of the fixed effect feed efficiency.

[3]Linear polynominal contrast: $*P \leq 0.05$.

[4]RASS, relative absorptive and secretory surface area (mucosal surface to serosal surface ratio). The RASS was only determined for the jejunum of low and high RFI female chickens.

819 **Table 4.** Least squares means of mucosal disaccharidase activities in mid jejunum of female and male broiler chickens of diverging residual feed

820 intake raised at two different locations

	Location 1				Location 2						
Enzyme activity (U/g protein)[1]	Low RFI	Medium RFI	High RFI	SEM	Low RFI	Medium RFI	High RFI	SEM	FE, P^2	Location, P^2	FE × location, P^2
Females											
Maltase	2240	2033	2448	163.8	1227	1527	1248	174.1	<0.001	0.096	0.51
Sucrase	123	188	231	19.4	213	168	135	20.6	<0.001	0.074	0.76
Lactase	9.6	7.7	7.4	1.84	9.1	8.7	8.2	1.96	0.81	0.91	0.44
Males											
Maltase	1989[b]	3208[a]	2755[ab]	290.5	1687	1397	1573	285.5	0.26	<0.001	0.038
Sucrase	209	252	254	31.7	172	161	169	31.1	0.77	0.008	0.64
Lactase	8.0	5.2	7.8	1.57	9.8	7.7	7.2	1.55	0.29	0.35	0.58

821 Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; FE, feed efficiency.

822 [1]Values are least squares means ± standard error of the mean (SEM).

823 [2]*P*: probability level of fixed effects feed efficiency, location and their two-way interaction.

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827 **Table 5.** Least squares means of mucosal expression of target genes in mid jejunum of female and male broiler chickens of diverging residual feed

828 intake raised at two different locations

	Location 1			Location 2					
Relative expression [1]	Low RFI	High RFI	SEM	Low RFI	High RFI	SEM	FE, P^2	Location, P^2	FE × location, P^2
Females									
CLDN1	1.076	1.157	0.1254	1.051	1.073	0.1343	0.70	0.68	0.82
CLDN5	1.504	2.180	0.7672	1.794	1.725	0.7990	0.70	0.92	0.64
ZO1	1.223	0.768	0.3595	1.765	1.562	0.3851	0.39	0.085	0.74
OCLN	1.007	0.694	0.2304	1.624	1.168	0.2468	0.12	0.031	0.77
IL1B	0.486	0.527	0.2068	0.562	1.399	0.2134	0.048	0.034	0.071
TNFA	6.723	1.442	3.1561	1.797	4.384	3.9548	0.71	0.78	0.27
TLR2	0.679	0.933	0.2890	0.766	1.359	0.3009	0.17	0.39	0.57
TLR4	0.537	0.625	0.1071	0.731	0.544	0.1154	0.66	0.62	0.23
IAP	1.018	0.819	0.2800	0.732	1.770	0.3539	0.21	0.32	0.070
MCT1	1.515	1.374	0.1975	1.493	1.272	0.2128	0.39	0.76	0.85
Males									
CLDN1	1.804	1.354	0.3673	1.678	1.085	0.3474	0.16	0.59	0.84
CLDN5	1.986	1.483	0.4553	2.012	1.387	0.4553	0.23	0.94	0.90
ZO1	0.806	0.700	0.1969	1.499	1.156	0.1979	0.26	0.007	0.55
OCLN	0.730	0.608	0.1677	1.196	1.000	0.1685	0.35	0.016	0.83
IL1B	0.707	0.361	0.2288	0.858	1.389	0.2436	0.70	0.019	0.074
TNFA	1.364	1.707	0.4333	3.080	2.301	0.5397	0.66	0.027	0.26
TLR2	0.617	0.464	0.2266	1.808	1.508	0.2266	0.33	<0.001	0.75
TLR4	0.734	0.670	0.0941	0.834	0.417	0.0969	0.017	0.43	0.074
IAP	0.730	0.759	0.2500	1.575	1.474	0.3062	0.90	0.010	0.82

MCT1	2.032	1.443	0.1828	1.729	0.989	0.1774	<0.001	0.044	0.68

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; FE, feed efficiency; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2]*P*: probability level of fixed effects feed efficiency, location and their two-way interaction.

834 **Table 6.** Least squares means of electrophysiological data and tissue permeability in distal
835 jejunum of female and male broiler chickens of diverging residual feed intake raised at
836 location 1

Item[1]	Low RFI	Medium RFI	High RFI	SEM	FE, $P^{2,3}$
Females					
I_{sc} (µA/cm^2)	23.0^{a}	18.8^{ab}	10.1^{b}	3.762	0.087*
G_T (ms/cm^2)	10.3^{a}	6.5^{b}	6.5^{b}	0.644	0.001**
J_{ms} of FITC (nmol/cm^2 × h)	0.119^{a}	0.035^{b}	0.049^{b}	0.021	0.030*
J_{ms} of HRP (pmol/cm^2 × h)	0.22	0.16	0.15	0.046	0.60
Males					
I_{sc} (µA/cm^2)	19.2	12.6	20.9	3.959	0.33
G_T (ms/cm^2)	8.23	6.41	8.03	0.975	0.38
J_{ms} of FITC (nmol/cm^2 × h)	0.126	0.065	0.082	0.023	0.19
J_{ms} of HRP (pmol/cm^2 × h)	0.41	0.27	0.27	0.093	0.47

837 Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); FE, feed efficiency; G_T, tissue
838 conductance; I_{sc}, short-circuit current; J_{ms}, mucosal-to-serosal flux; RFI, residual feed intake.
839 [1]Values are least squares means ± standard error of the mean (SEM).
840 [2]*P*: probability level of the fixed effect feed efficiency.
841 [3]Linear polynominal contrast: *$P \leq 0.05$, **$P < 0.001$.
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Supplementary Material

Composition of buffers used in the Ussing Chamber experiment

Transport and rinsing buffers contained the following chemicals (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany, in mmol/L): 115 NaCl, 25 $NaHCO_3$, 5 KCl, 2.4 Na_2HPO_4, 0.4 NaH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$ and 5 D-glucose; pH 7.4). Buffers were gassed with carbogen gas before the start of the Ussing chamber run each morning and kept at 4°C. The experimental buffer contained (mmol/L): 115 NaCl, 25 $NaHCO_3$, 20 mannitol, 5 KCl, 5 HEPES, 2.4 Na_2HPO_4, 0.4 NaH_2PO4, 1.2 $CaCl_2$, 1.2 $MgCl_2$ and 0.172 kanamycin sulphate; pH 7.4. To nurture the jejunal tissue during the incubation, 100 µmol glucose was added to the serosal side of the chamber, resulting in a final concentration of 10 mM.

Supplementary Table 1. Ingredients and chemical composition of diets

Item	Starter[1]	Grower[2]	Finisher[3]
Ingredient (g/kg as-fed)			
Corn	612	660	679
Soybean meal	331	282	260
Soybean oil	17.5	20.6	27.7
Limestone flour	11.0	9.8	7.0
Salt	2.0	2.0	2.3
Dicalcium phosphate	16.1	15.0	13.4
Vitamin/mineral-premix	11.0	11.0	10.0
Analyzed chemical composition at L1			
Dry matter (g/kg)	926	923	914
Crude protein (g/kg DM)	243	223	216
Ether extracts (g/kg DM)	50	52	59
Crude fiber (g/kg DM)	31	27	28
Crude ash (g/kg DM)	69	62	55
Starch (g/kg DM)	462	506	514
Sugar (g/kg DM)	40	46	49
Calcium (g/kg DM)	11.9	10.7	8.9
Phosphorus (g/kg DM)	8.2	7.8	6.9
Analyzed chemical composition at L2			
Dry matter (g/kg)	908	902	902
Crude protein (g/kg DM)	221	219	209
Crude ash (g/kg DM)	94	81	72
Metabolizable energy[4] (kcal/kg)	3,272.8	3,416.1	3,487.8

[1]Premix provided per kilogram of starter diet: vitamin A as retinyl acetate, 13,000 IU; vitamin D_3 as cholecalciferol, 5,000 IU; vitamin E as alpha-tocopherol acetate, 80 IU; vitamin K, 3 mg; thiamin, 3 mg; riboflavin, 9 mg; pyridoxine, 4 mg; vitamin $B_{12,}$ 20 μg; biotin, 0.15 mg; calcium pantothenate, 15 mg; nicotinic acid, 60 mg; folic acid, 2 mg; 500 mg choline chloride; methionine, 3,405 mg; threonine, 745 mg; lysine, 2,812 mg; I, 1 mg as calcium iodate; Se, 0.35 mg as sodium selenite; Fe, 40 mg as ferrous sulphate; Mo, 0.5 mg as sodium molybdate; Mn, 100 mg as manganous oxide; Cu, 15 mg as copper sulfate; Zn, 100 mg as zinc oxide.

[2]Premix provided per kilogram of grower diet: vitamin A as retinyl acetate, 10,000 IU; vitamin D_3 as cholecalciferol, 5,000 IU; vitamin E as alpha-tocopherol acetate, 50 IU; vitamin K, 3 mg; thiamin, 2 mg; riboflavin, 8 mg; pyridoxine, 3 mg; vitamin $B_{12,}$ 15 μg; biotin, 0.12 mg; calcium pantothenate, 12 mg; nicotinic acid, 50 mg; folic acid, 2 mg; 400 mg choline chloride; methionine, 3,018 mg; threonine, 726 mg; lysine, 2,831 mg; I, 1 mg as calcium iodate; Se, 0.35 mg as sodium selenite; Fe, 40 mg as ferrous sulphate; Mo, 0.5 mg as sodium molybdate; Mn, 100 mg as manganous oxide; Cu, 15 mg as copper sulfate; Zn, 100 mg as zinc oxide.

[3]Premix provided per kilogram of finisher diet: vitamin A as retinyl acetate, 10,000 IU; vitamin D_3 as cholecalciferol, 5,000 IU; vitamin E as alpha-tocopherol acetate, 50 IU; vitamin K, 3 mg; thiamin, 2 mg; riboflavin, 6 mg; pyridoxine, 3 mg; vitamin $B_{12,}$ 15 μg; biotin, 0.12 mg; calcium pantothenate, 10 mg; nicotinic acid, 50 mg; folic acid, 1 mg; 350 mg choline chloride; methionine, 2,514 mg; threonine, 361 mg; lysine, 1,779

mg; I, 1 mg as calcium iodate; Se, 0.35 mg as sodium selenite; Fe, 40 mg as ferrous sulphate; Mo, 0.5 mg as sodium molybdate; Mn, 100 mg as manganous oxide; Cu, 15 mg as copper sulfate; Zn, 100 mg as zinc oxide.

[4]Calculated according to NRC (1994).

Supplementary Table 2. Oligonucleotide primers for target and housekeeping genes

Gene symbol[1]	Accession number[2]	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	Eff (%)[3]	Corr[4]
ACTB	NM_205518.1	Gallus gallus actin, beta	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA	152	95.1	0.999
B2M	NM_001001750.1	Gallus gallus beta-2-microglobulin	ACTTCACACCCAGCAGCG	GGCACAGCTCAGAACTCGG	103	90.2	0.999
GAPDH	NM_204305.1	Gallus gallus glyceraldehyde-3-phosphate dehydrogenase	TATCTTCCAGGAGCGTGACC	TCTCCATGGTGGTGAAGACA	95	91.3	1.000
HPRT1	NM_204848.1	Gallus gallus hypoxanthine phosphoribosyltransferase 1	GATGAACAAGGTTACGACCTGGA	TATAGCCACCCTTGAGTACACAGAG	181	104.6	0.998
OAZ1	NM_204916.1	Gallus gallus ornithine decarboxylase antizyme 1	GCGAGGGAATAGTCAGAGGG	GCCTTGACTGGACATTCAGAATT	125	95.9	0.999
SNRPD3	NM_001007838.1	Gallus gallus small nuclear ribonucleoprotein D3 polypeptide	CCAGATATGTTGAAGAACGCTCCT	GTTGCCACGACCCATACCA	141	94.4	0.998
OCLN	NM_205128.1	Gallus gallus occludin	CAGCACCTACCTCAACCAGTACAT	AGGCAGAGCAGGATGACGAT	102	95.6	0.999
TJP1 (ZO1)	XM_015278975.1, XM_015278976.1, XM_015278977.1, XM_015278978.1, XM_015278979.1, XM_015278980.1, XM_015278981.1,	Gallus gallus tight junction protein 1 (zona occludens 1)	CAACTGGTGTGGGTTTCTGAA	TCACTACCAGGAGCTGAGAGGTAA	101	96.0	0.999
CLDN1	NM_001013611.2	Gallus gallus claudin 1	CCAGGTGAAGAAGATGCGGA	GGTGTGAAAGGGTCATAGAAGGC	129	106.0	0.998
CLDN5	NM_204201.1	Gallus gallus claudin 5	CAGGTCGCCAGAGATACAGG	GAAGCCTCTCATAGCCTAAGCATC	157	97.5	0.999
ALPI	XM_015291488.1, XM_015291489.1	Gallus gallus alkaline phosphatase, intestinal	CCACTCGCATGTCTTCACCTT	CATTGCCGTAGAGGATGCTG	116	91.1	1.000
TLR2	NM_001161650.1, NM_204278.1	Gallus gallus toll-like receptor 2	GGGCACAGGTTGGGAGTG	CCAACGACCACCAGGATGA	112	90.5	0.998
TLR4	NM_001030693.1	Gallus gallus toll-like receptor 4	TGCAGTTTCTGGATCTTTCAAG	GCGACGTTAAGCCATGGAA	144	107.7	0.998
IL1B	NM_204524.1	Gallus gallus interleukin 1, beta	GGGCATCAAGGGCTACAA	CTGTCCAGGCGGTAGAAGAT	88	90.7	0.999
TNFA	AY765397.1	Gallus gallus TNF-alpha factor	TCGGGAGTGGGCTTTAAGAAG	GAAAGCCACTAGGAGCAGACA	112	90.4	0.997
SLC16A1 (MCT1)	NM_001006323.1, XM_015298930.1	Gallus gallus solute carrier family 16 (monocarboxylate transporter), member 1 (SLC16A1)	CTTTGGCTGGCTTAGCTCG	TTGTAGTCACCATACATGTCGTTGA	162	99.8	0.998

[1]Alternate gene names are shown in brackets.

[2]National Center for Biotechnology Information (NCBI) Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

[3]Eff, PCR efficiency: $E = 10^{(-1/slope)}-1$.

[4]Corr, Correlation coefficient of standard curve.

Supplemental Table 3. Least squares means of feed efficiency metrics, total feed intake, total body weight gain and body weight of female and male broiler chickens of diverging residual feed intake raised at two different locations[1]

	Location 1				Location 2						
Item[2]	Low RFI	Medium RFI	High RFI	SEM	Low RFI	Medium RFI	High RFI	SEM	FE, *P*[3,4]	location, *P*[3]	FE × location, *P*[3]
Females											
RFI (g)	-195	18	97	27.5	-267	-3	232	29.2	<0.001**	0.41	0.20
RBG (g)	-2.1	0.5	1.3	5.65	0.2	1.5	2.1	6.01	0.90	0.78	0.99
RIG (g)	193	-18	-196	27.6	267	4	-231	29.3	<0.001**	0.38	0.20
FCR (g/g)	1.46	1.55	1.62	0.026	1.65	1.71	1.89	0.028	<0.001**	<0.001	0.11
TFI, d 7-36 of life (g)	3,334	3,510	3,751	123.2	3,559	3,461	3,797	131.0	0.027*	0.48	0.57
TBWG, d 7-36 of life (g)	2,251	2,220	2,279	67.7	1,966	1,865	1,856	72.0	0.65	<0.001	0.64
BW, d 36 of life (g)	2,392	2,359	2,420	68.9	2,115	2,015	2,009	73.2	0.65	<0.001	0.67
BW at euthanization (g)	2,851	2,840	2,861	89.3	2,584	2,574	2,594	94.9	0.97	0.001	1.00
Males											
RFI (g)	-183	6	303	31.1	-211	-6	231	30.6	<0.001**	0.15	0.61
RBG (g)	6.8	1.8	10.4	6.27	4.2	-3.9	-2.7	6.16	0.55	0.17	0.69
RIG (g)	190	-4	-292	31.3	215	2	-234	30.8	<0.001**	0.25	0.70
FCR (g/g)	1.4	1.48	1.61	0.028	1.58	1.69	1.79	0.027	<0.001**	<0.001	0.77
TFI, d 7-36 of life (g)	3,682	3,901	4185	99.9	3823	3857	4321	98.2	<0.001**	0.34	0.57
TBWG, d 7-36 of life (g)	2,573	2,593	2,615	77.7	2,228	2,083	2,214	76.4	0.58	<0.001	0.560
BW, d 36 of life (g)	2,712	2,733	2,756	79.0	2,380	2,233	2,367	79.4	0.58	<0.001	0.560
BW at euthanization (g)	3,036	2,989	3,070	109.0	3,043	2,923	3,081	107.0	0.54	0.86	0.93

45 Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; BW, body weight; FCR, feed
46 conversion ratio; FE, feed efficiency; RBG, residual body weight gain; RFI, residual feed intake; RIG, residual feed intake and body weight gain; TBWG, total body weight gain;
47 TFI, total feed intake.
48 [1]Metzler-Zebeli, B. U., E. Magowan, M. Hollmann, M. E. E. Ball, A. Molnár, P. G. Lawlor, R. J. Hawken, N. E. O'Connell, and Q. Zebeli (2017) Assessing serum metabolite
49 profiles as biomarkers for feed efficiency in broiler chickens reared at geographically distant locations. Submitted to Poultry Science.
50 [2]Values are least squares means ± standard error of the mean (SEM).
51 [3]*P*: probability level of fixed effects feed efficiency, location and their two-way interaction.
52 [4]Linear polynominal contrast: *$P \leq 0.05$; **$P \leq 0.001$.
53
54

Supplementary Table 4. Least squares means of morphological characteristics of different intestinal segments in male chickens of diverging residual feed intake raised at location 1

Item[1]	Low RFI	Medium RFI	High RFI	SEM	FE, *P*
Duodenum					
Villus height (µm)	1,626	1,648	1,617	57.4	0.93
Villus width (µm)	182	179	176	6.5	0.85
Crypt depth (µm)	149	153	153	6.5	0.89
Muscle layer (µm)	204	217	222	14.9	0.69
Villus height:crypt depth ratio	11.1	10.9	10.7	0.54	0.86
Apparent villus surface area (mm^2)	0.92	1.00	0.94	0.063	0.65
Goblet cells per 250 µm villus surface	7.1	8.3	7.8	0.45	0.19
Intraepithelial lymphocytes per 400 µm villus surface	8.9	9.0	8.6	0.89	0.89
Jejunum					
Villus height (µm)	982	1,045	986	37.5	0.43
Villus width (µm)	149	168	152	11.0	0.44
Crypt depth (µm)	109	116	107	4.3	0.31
Muscle layer (µm)	187	223	202	14.2	0.23
Villus height:crypt depth ratio	9.1	9.0	9.2	0.34	0.90
Apparent villus surface area (mm^2)	0.46	0.56	0.47	0.049	0.30
Goblet cells per 250 µm villus surface	11.0	10.7	10.1	0.65	0.56
Intraepithelial lymphocytes per 400 µm villus surface	11.0	12.2	10.7	0.76	0.32
Ileum					
Villus height (µm)	728	722	664	33.7	0.35
Villus width (µm)	148	143	134	5.7	0.27
Crypt depth (µm)	99	106	102	4.5	0.56
Muscle layer (µm)	226	265	234	18.4	0.30
Villus height:crypt depth ratio	7.2	6.9	6.7	0.28	0.54
Apparent villus surface area (mm^2)	0.34	0.33	0.28	0.022	0.23
Goblet cells per 250 µm villus surface	14.5	15.4	15.5	0.78	0.61
Intraepithelial lymphocytes per 400 µm villus surface	10.5	10.3	10.5	0.98	0.97
Ceca					
Crypt depth (µm)	311	322	316	23.4	0.94
Goblet cells per 250 µm villus surface	5.9	5.6	6.3	0.65	0.75

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); FE, feed efficiency; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2]*P*: probability level of the fixed effect feed efficiency.

Supplementary Table 5. Selected Pearson's correlation coefficients for RFI, RBG, RIG, FCR, TFI and TBWG with intestinal variables of female chickens of diverging RFI raised at two locations

Item		TFI	TBWG	RFI	RBG	RIG	FCR
Length (cm/kg BW)							
Duodenum	*r* =	-0.25	-0.49	0.08	0.08	-0.07	0.34
	P =	0.069	<0.001	0.60	0.58	0.63	0.015
	n =	52	52	52	52	52	52
Jejunum	*r* =	-0.35	-0.21	0.09	0.07	-0.08	-0.14
	P =	0.010	0.14	0.53	0.60	0.56	0.33
	n =	52	52	52	52	52	52
Ileum	*r* =	-0.05	-0.58	0.04	0.08	-0.03	0.69
	P =	0.73	<0.001	0.80	0.56	0.84	<0.001
	n =	52	52	52	52	52	52
Cecum	*r* =	-0.20	-0.65	-0.02	-0.11	0.01	0.60
	P =	0.15	<0.001	0.88	0.44	0.93	<0.001
	n =	52	52	52	52	52	52
Colon	*r* =	-0.30	-0.48	0.02	-0.01	-0.02	0.25
	P =	0.030	<0.001	0.87	0.96	0.87	0.08
	n =	52	52	52	52	52	52
Weight (g/kg BW)							
Duodenum	*r* =	-0.13	-0.29	-0.01	0.29	0.03	0.22
	P =	0.34	0.039	0.96	0.040	0.82	0.13
	n =	52	52	52	52	52	52
Jejunum	*r* =	-0.10	0.21	0.07	0.09	-0.07	-0.36
	P =	0.48	0.14	0.60	0.54	0.64	0.009
	n =	52	52	52	52	52	52
Ileum	*r* =	0.02	-0.51	0.00	0.05	0.00	0.68
	P =	0.87	<0.001	1.00	0.74	0.98	<0.001
	n =	52	52	52	52	52	52
Cecum	*r* =	-0.033	-0.542	-0.080	-0.023	0.078	0.654
	P =	0.82	<0.001	0.57	0.87	0.58	<0.001
	n =	52	52	52	52	52	52
Colon	*r* =	-0.17	-0.38	0.07	-0.03	-0.07	0.29
	P =	0.23	0.006	0.63	0.83	0.61	0.035
	n =	52	52	52	52	52	52
Retention (%)							
DM	*r* =	0.18	-0.34	0.21	0.12	-0.20	0.67
	P =	0.21	0.014	0.1	0.40	0.16	<0.001
	n =	52	52	52	52	52	52
CP	*r* =	0.04	-0.46	0.03	0.14	-0.02	0.64
	P =	0.80	<0.001	0.82	0.32	0.88	<0.001
	n =	52	52	52	52	52	52

Excretion (g/day)							
DM	*r* =	0.266	0.652	0.017	-0.021	-0.019	-0.520
	P =	0.057	<0.001	0.90	0.88	0.89	<0.001
	n =	52	52	52	52	52	52
Ash	*r* =	0.39	0.27	-0.03	0.01	0.03	0.10
	P =	0.005	0.054	0.82	0.97	0.82	0.50
	n =	52	52	52	52	52	52
CP	*r* =	0.21	0.54	0.16	-0.06	-0.16	-0.43
	P =	0.13	<0.001	0.27	0.67	0.25	0.002
	n =	52	52	52	52	52	52
Jejunal expression							
IL1B	*r* =	0.04	-0.46	0.40	0.10	-0.39	0.52
	P =	0.82	0.013	0.036	0.60	0.039	0.005
	n =	30	30	30	30	30	30
TNFA	*r* =	0.36	0.49	-0.06	-0.05	0.06	-0.06
	P =	0.075	0.014	0.76	0.81	0.77	0.76
	n =	30	30	30	30	30	30
TLR2	*r* =	-0.21	-0.55	0.37	0.11	-0.36	0.33
	P =	0.29	0.003	0.055	0.58	0.060	0.088
	n =	30	30	30	30	30	30
ZO1	*r* =	0.267	-0.104	0.095	0.134	-0.087	0.408
	P =	0.15	0.58	0.62	0.48	0.65	0.025
	n =	30	30	30	30	30	30
Enzyme activity (U/g protein)							
Maltase	*r* =	-0.05	0.27	0.04	0.08	-0.03	-0.42
	P =	0.72	0.051	0.78	0.58	0.81	0.002
	n =	52	52	52	52	52	52
Sucrase	*r* =	-0.22	0.06	0.03	0.03	-0.03	-0.35
	P =	0.13	0.68	0.82	0.82	0.84	0.012
	n =	52	52	52	52	52	52

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; BW, body weight; CP, crude protein; DM, dry matter; FCR, feed conversion ratio; RBG, residual body weight gain; RFI, residual feed intake; RIG, residual feed intake and body weight gain.

Supplementary Table 6. Selected Pearson's correlation coefficients for RFI, RBG, RIG, FCR, TFI and TBWG with intestinal variables of male chickens of diverging RFI raised at two locations

Item		TFI	TBWG	RFI	RBG	RIG	FCR
Length (cm/kg BW)							
Duodenum	*r* =	-0.26	-0.52	-0.06	-0.12	0.05	0.39
	P =	0.053	<0.001	0.68	0.39	0.74	0.004
	n =	55	55	55	55	55	55
Ileum	*r* =	0.10	-0.44	-0.08	-0.19	0.06	0.62
	P =	0.47	<0.001	0.56	0.16	0.65	<0.001
	n =	55	55	55	55	55	55
Cecum	*r* =	-0.08	-0.49	-0.06	-0.28	0.03	0.53
	P =	0.56	<0.001	0.67	0.038	0.80	<0.001
	n =	55	55	55	55	55	55
Colon	*r* =	-0.03	-0.42	0.00	-0.27	-0.02	0.50
	P =	0.84	0.001	0.98	0.048	0.89	<0.001
	n =	55	55	55	55	55	55
Weight (g/kg BW)							
Duodenum	*r* =	0.25	-0.07	0.17	-0.11	-0.18	0.33
	P =	0.061	0.61	0.22	0.41	0.20	0.013
	n =	55	55	55	55	55	55
Jejunum	*r* =	0.19	-0.39	-0.04	-0.17	0.03	0.65
	P =	0.17	0.004	0.77	0.20	0.86	<0.001
	n =	55	55	55	55	55	55
Ileum	*r* =	0.08	-0.43	-0.12	-0.14	0.11	0.59
	P =	0.58	0.001	0.38	0.32	0.43	<0.001
	n =	55	55	55	55	55	55
Cecum	*r* =	0.13	-0.30	0.03	-0.23	-0.05	0.49
	P =	0.34	0.027	0.82	0.086	0.71	<0.001
	n =	55	55	55	55	55	55
Colon	*r* =	0.02	-0.36	-0.01	-0.24	-0.01	0.48
	P =	0.91	0.006	0.94	0.081	0.94	<0.001
	n =	55	55	55	55	55	55
Retention (%)							
CP	*r* =	-0.04	-0.43	-0.14	-0.22	0.12	0.50
	P =	0.75	<0.001	0.30	0.10	0.37	<0.001
	n =	55	55	55	55	55	55
Ash	*r* =	0.18	-0.10	0.07	-0.11	-0.08	0.33
	P =	0.18	0.46	0.63	0.42	0.58	0.015
	n =	55	55	55	55	55	55
Excretion (g/day)							
DM	*r* =	0.38	0.58	0.34	0.30	-0.32	-0.35
	P =	0.005	<0.001	0.010	0.027	0.019	0.008
	n =	55	55	55	55	55	55

CP	*r* =	0.18	0.46	0.33	0.27	-0.30	-0.40
	P =	0.19	<0.001	0.014	0.046	0.024	0.003
	n =	55	55	55	55	55	55
Jejunal expression							
CLDN1	*r* =	-0.14	0.24	-0.20	0.14	0.22	-0.39
	P =	0.42	0.17	0.24	0.43	0.22	0.022
	n =	36	36	36	36	36	36
OCLN	*r* =	0.12	-0.18	0.06	0.17	-0.05	0.33
	P =	0.48	0.28	0.74	0.33	0.79	0.048
	n =	36	36	36	36	36	36
ZO1	*r* =	0.14	-0.17	0.10	0.10	-0.09	0.33
	P =	0.40	0.32	0.56	0.54	0.60	0.052
	n =	36	36	36	36	36	36
MCT1	*r* =	-0.34	0.11	-0.31	0.09	0.32	-0.45
	P =	0.044	0.54	0.062	0.60	0.057	0.006
	n =	36	36	36	36	36	36
TLR4	*r* =	-0.34	-0.19	-0.20	0.25	0.22	-0.08
	P =	0.040	0.26	0.23	0.14	0.19	0.63
	n =	36	36	36	36	36	36

71 Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences
72 Institute, Hillsborough, UK; BW, body weight; CP, crude protein; DM, dry matter; FCR, feed conversion ratio;
73 RBG, residual body weight gain; RFI, residual feed intake; RIG, residual feed intake and body weight gain.
74

Supplementary Table 7. Pearson's correlation coefficients for retention and excretion of nutrients and intestinal length and weight of males and female chickens of diverging residual feed intake raised at two locations

Item		Retention (%)			Excretion (g/day)		
		DM	CP	Ash	DM	Ash	CP
Females							
Length (cm/kg BW)							
Duodenum	*r* =	0.30	0.34	0.13	-0.35	-0.12	-0.26
	P =	0.030	0.014	0.36	0.011	0.41	0.059
	n =	52	52	52	52	52	52
Jejunum	*r* =	-0.21	-0.27	-0.26	0.13	-0.27	0.28
	P =	0.13	0.050	0.068	0.36	0.057	0.048
	n =	52	52	52	52	52	52
Ileum	*r* =	0.68	0.87	0.59	-0.76	0.27	-0.83
	P =	<0.001	<0.001	<0.001	<0.001	0.053	<0.001
	n =	52	52	52	52	52	52
Colon	*r* =	0.28	0.32	0.29	-0.29	0.12	-0.33
	P =	0.043	0.019	0.035	0.035	0.41	0.018
	n =	52	52	52	52	52	52
Cecum	*r* =	0.58	0.74	0.37	-0.71	0.10	-0.69
	P =	<0.001	<0.001	0.007	<0.001	0.48	<0.001
	n =	52	52	52	52	52	52
Weight (g/kg BW)							
Duodenum	*r* =	0.28	0.33	0.23	-0.26	0.18	-0.30
	P =	0.045	0.018	0.096	0.062	0.20	0.031
	n =	52	52	52	52	52	52
Jejunum	*r* =	-0.40	-0.49	-0.39	0.45	-0.15	0.51
	P =	0.004	<0.001	0.004	<0.001	0.29	<0.001
	n =	52	52	52	52	52	52
Ileum	*r* =	0.61	0.84	0.52	-0.73	0.33	-0.80
	P =	<0.001	<0.001	<0.001	<0.001	0.016	<0.001
	n =	52	52	52	52	52	52
Cecum	*r* =	0.62	0.84	0.46	-0.72	0.26	-0.76
	P =	<0.001	<0.001	<0.001	<0.001	0.060	<0.001
	n =	52	52	52	52	52	52
Colon	*r* =	0.37	0.41	0.27	-0.30	0.13	-0.30
	P =	0.007	0.002	0.055	0.030	0.34	0.031
	n =	52	52	52	52	52	52
Males							
Length (cm/kg BW)							
Duodenum	*r* =	0.38	0.39	0.24	-0.42	-0.15	-0.38
	P =	0.004	0.004	0.080	0.001	0.29	0.005
	n =	55	55	55	55	55	55
Jejunum	*r* =	-0.05	-0.22	-0.02	-0.04	-0.42	0.09
	P =	0.72	0.10	0.90	0.77	0.001	0.45
	n =	55	55	55	55	55	55
Ileum	*r* =	0.62	0.84	0.38	-0.61	0.42	-0.75
	P =	<0.001	<0.001	0.004	<0.001	0.002	<0.001
	n =	55	55	55	55	55	55
Cecum	*r* =	0.52	0.66	0.18	-0.55	0.15	-0.56
	P =	<0.001	<0.001	0.18	<0.001	0.29	<0.001

	n =	55	55	55	55	55	55
	r =	0.37	0.52	0.22	-0.38	0.30	-0.44
Colon	*P* =	0.005	<0.001	0.12	0.004	0.028	0.001
	n =	55	55	55	55	55	55
Weight (g/kg BW)							
	r =	0.22	0.29	0.27	-0.08	0.35	-0.19
Duodenum	*P* =	0.11	0.029	0.043	0.58	0.010	0.17
	n =	55	55	55	55	55	55
	r =	-0.17	-0.32	-0.05	0.30	-0.17	0.38
Jejunum	*P* =	0.23	0.018	0.72	0.028	0.20	0.004
	n =	55	55	55	55	55	55
	r =	0.56	0.79	0.37	-0.49	0.52	-0.65
Ileum	*P* =	<0.001	<0.001	0.006	<0.001	<0.001	<0.001
	n =	55	55	55	55	55	55
	r =	0.55	0.79	0.30	-0.56	0.39	-0.66
Cecum	*P* =	<0.001	<0.001	0.027	<0.001	0.003	<0.001
	n =	55	55	55	55	55	55
	r =	0.21	0.43	0.10	-0.21	0.46	-0.31
Colon	*P* =	0.13	0.001	0.48	0.13	<0.001	0.021
	n =	55	55	55	55	55	55

78 Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences

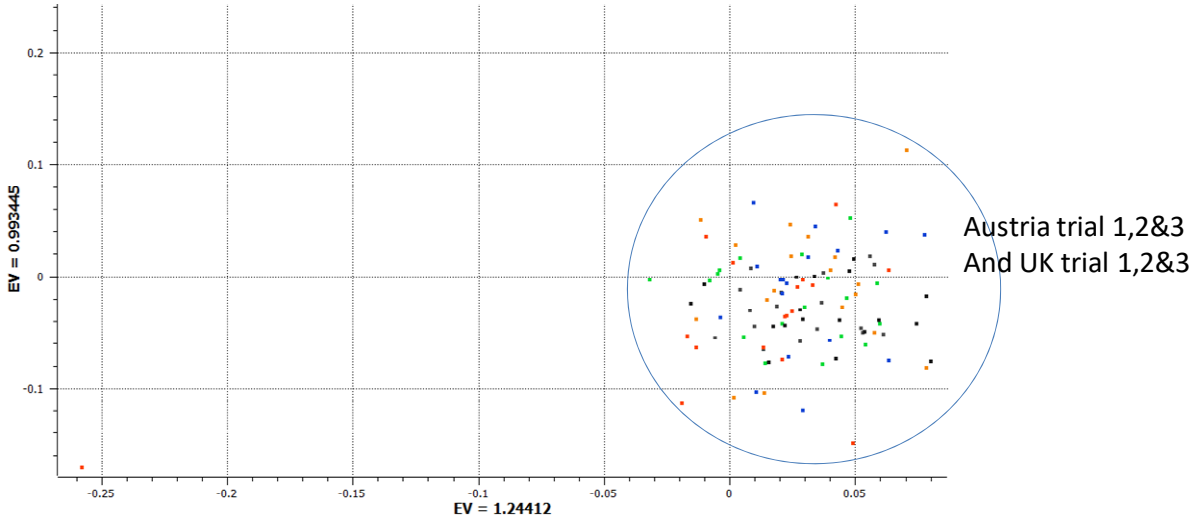
79 Institute, Hillsborough, UK; BW, body weight; CP, crude protein; DM, dry matter.

80

Genomic and relationship analysis

Single nucleotide polymorphism (SNP) genotypes were used to examine the genetic relationship of all birds within and between each population received.

Simple genotype principle components analysis (PCA) on about 4000 SNP from each batch of genotypes taken from samples at both the location 1 (Austria) and location 2 (UK) were performed. Comparisons were made with known Cobb500 chickens, Cobb chickens produced with a line difference, and with breeder parents to ensure that all chicks were of Cobb500 genetics which was confirmed by the PCA. In Supplementary Figure 1, the PCA plot indicates that all chicks were of the same genotype. Breed anomalies can therefore not explain observed location-associated differences in intestinal length and weight and nutrient retention.



Supplementary Figure 1. Principal component analysis plot of single nucleotide polymorphisms from all Cobb500 chickens used at location 1 (Austria) and location 2 (UK). 1, 2, 3 indicates the batch number at each location.

In order to achieve the genetic relationship of each pair of samples supplied, a G-matrix was established using the PreGS program by Prof I. Misztal (Animal Breeding and Genetics

group, University of Georgia, Athens, GA, USA). Supplemental Table 8 lists the relationship statistic per population.

These data indicate that there is very little genetic relationship between any two birds within replicate batch 1 and replicate batch 2 from the location 1. In replicate batch 3 at location 1, two birds appeared to be half-sibs (relationship of 0.25). Similarly, the replicate batch 1 from location 2 appeared to contain two birds that are half-sibs (relationship of 0.20). The overall relationships within and between populations has been plotted and is illustrated in Supplemental Figure 1.

Supplementary Table 8. Genomic relationships among chickens

	comparisons	genomic relationships among birds			
		mean	sd	min	max
Location 1 + 2	2415	0	0.02	-0.05	0.25

Supplementary Figure 2. G-relationships among chickens from both locations